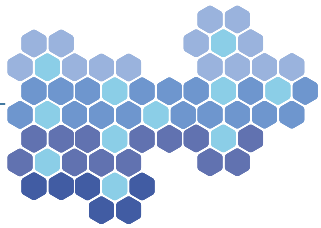

Gold Standard

Advances in molecular biology techniques have so far received little attention in clinical microbiology labs. However, using a novel extraction system that provides stable pools of DNA and RNA in just two steps could facilitate adoption of these techniques

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Since the discovery of polymerase chain reaction (PCR) in the late 1980s, research has been applied to introduce molecular solutions in routine clinical microbiology, and molecular biology methods continue to rapidly evolve. However, the majority of diagnostics still rely on classical analytical tools. Molecular biology currently offers a wide repertoire of techniques and permutations of these tools.

Nucleic acids can provide key biomarkers in the diagnostic field as a measurable characteristic of an organism that can reflect the physiological state, and can also be an indicator of the presence of a disease. More recently, techniques have been developed using biomarkers to assess a patient's response to a treatment and in predicting drug resistance. This is achieved by using profiles of transcripts of the genome (in particular, messenger RNA [mRNA] and microRNA [miRNA]) as biomarkers in molecular *in vitro* diagnostics. RNA specifically integrates genetic and epigenetic mechanisms of gene regulation, and its expression reflects the state of biological systems.

Keywords

Molecular biology
Plasmodium detection
RNA extraction
RTqPCR

However, while development of these techniques has swiftly progressed in research laboratories, there still remains limited use in routine clinical microbiology labs. There are two key reasons for this. Firstly, for an investigator to perform reliable molecular diagnostic (quantitative reverse transcription PCR [RTqPCR] or quantitative PCR [qPCR]) or downstream recombinant DNA technology assays from microbiological clinical samples, DNA and RNA isolation is time-consuming and becomes a bottleneck. Secondly, the extraction of stable RNA pools is particularly challenging as the resistance to digestion is critical and the most rapid RNA isolation methods can take 30-60 minutes, which means some digestion may have been initiated. The process often requires many steps and the increased hands-on time may also introduce the potential for sample mix-up or further loss due to intrinsic degradation.

In this study, a novel extraction system – in which a pool of stable RNA can be achieved in two very simple steps and would be suitable for use in clinical microbiological labs – was tested to demonstrate protection of pure RNA isolated from whole blood. The isolated RNA was extracted using a gold standard purification system and was added to the novel extraction system, and a ribonuclease (RNase) enzyme was added to induce digestion. A semi-clinical test against this type of degradation was also carried out on extracts obtained from malaria-infected blood samples. The preliminary results offer a solution to the current bottleneck observed in the diagnostic lab setting, should it be introduced in routine clinical microbiology.

Experimental Methods

Recently there have been developments in a novel extraction system, which increase the stability of the most labile nucleic acids. In order to determine the effectiveness of this system, the RNA protection obtained by addition of the novel extraction solution to isolated and clean RNA extracts was tested by adding an RNase enzyme to the sample and testing the resistance to this specific degradation.

In a parallel set of assays, the study was extended to measure resistance to RNase-specific degradation using clinical samples – real malaria-infected blood and compared against the gold standard system currently used for detecting malaria. In both experiments, the degradation was measured by using a qPCR master mix, which amplified genomic DNA and an RTqPCR, which converted RNA to complementary DNA (cDNA) and measured both DNA and cDNA.

Results and Discussion

After adding the RNase A to clean RNA extracts obtained from the gold standard system with and without the presence of the novel extraction system, the protection obtained was observed in the threshold cycle values shown in Table 1. These results clearly indicate that the presence

		(RT-qPCR mix)	(qPCR mix)
Isolated RNA extract plus water	Without RNase A	**24.07, 24.9, 24.76**	29.79, 29.09, 29.76
	With RNase A	29.6, 29.9, 30.01	29.31, 29.01, 29.03
Isolated RNA extract plus novel extraction system	Without RNase A	**24.33, 23.9, 24.9**	29.99, 29.89, 29.05
	With RNase A	**24.68, 24.03, 25.01**	29.87, 29.31, 29.02

Table 1: Data from three independent repeats after incubation with 1IU of freshly prepared, pure RNase A at 37°C for 1 hour

	(RT-qPCR mix)			(qPCR mix)		
	Without RNase A	RNase A 1h	RNase A 1.5h	Without RNase A	Without RNase A 1h	RNase A 1.5h
Novel extraction	18.78, 19.01	**20.6, 19.67**	**20.9, 20.2**	25.71, 25.6	25.96, 25.73	26.1, 26.23
Gold standard extraction	17.24, 17.09	25.59, 26.01	25.99, 26.7	25.42, 25.55	25.81, 26.06	26.81, 26.93

Table 2: Data from two independent repeats after incubation with 1IU of freshly prepared, pure RNase A at 37°C for 1 and 1.5 hours

of the active principle used by this new approach protects RNAs from enzymatic degradation.

Parallel testing using clinical samples (malaria-infected blood samples) also confirmed the resistance to RNase-specific degradation with the addition of the novel extraction system versus extracts from a gold standard system (see Table 2). In this experiment, the infected whole blood was added directly to the novel extraction system and processed in less than three minutes compared to the gold standard, which took over 60 minutes.

The experiments performed show that, at this stage, the addition of the novel extraction system – when compared to the standardised gold standard extracts – specifically protects and prevents the digestion of the RNA. Under the same conditions, a pure extract of RNA using the gold standard is completely degraded after one hour of incubation. This trend is maintained when the presence of the digestion enzyme is upheld for longer and the RNA in the novel extraction system remains undigested even after 90 minutes of incubation with RNase A.

Since all these data were obtained through the amplification of *plasmodium* from clinical samples infected with the parasite by RTqPCR and PCR, it could be argued that a new strategy to increase the performance of *plasmodium* detection by RTqPCR may be possible due to the protective nature of the novel extraction system.

As reported by a number of authors, there is a need for a technical solution that allows the extraction of stable RNAs so as to perform 18S ribosomal RNA transcript-based detection for increased sensitivity and better diagnostics (1-3). Introduction of RTqPCR as a routine molecular technique in microbiological diagnostics depends only on the generation of stable RNA linked to a RTqPCR. In fact, it has been reported by the same authors that the molecular detection of low levels of gametocyte-specific mRNA would enable identification to be 4-10 times higher than estimated by microscopy. However, the labile nature of RNA and the ubiquitous presence of RNases pose challenges that are still to be overcome.

Further Testing

Data from the experiments described in this paper prove the feasibility of a new strategy for *plasmodium* detection: coupling a conservative retro-transcription of all mRNA targets available with a subsequent qPCR of the retro-transcribed cDNAs obtained through a novel extraction protocol. This approach, which provides stability and protection to degradation shown by those extracts, will require further testing against different degradative agents in order to ensure the robustness that would be necessary for any test to potentially be used in clinic. It does suggest, however, that the two key reasons for not adopting molecular techniques in clinical microbiological labs are on the way to being satisfied.

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